REPORTS

Relationship Between Environmental Tobacco Smoke Exposure and Carcinogen–Hemoglobin Adduct Levels in Nonsmokers

*S. Katharine Hammond, Jacalyn Coghlin, Peter H. Gann, Maureen Paul, Koli Taghizadeh, Paul L. Skipper, Steven R. Tannenbaum**

Background: **A potent bladder carcinogen for workers in the dye industry, 4-aminobiphenyl (4-ABP), is present in environmental tobacco smoke and has been shown to bond covalently with hemoglobin.** ***Purpose:*** **The goal of this study was to examine the relationship between exposure to environmental tobacco smoke and levels of 4-ABP–hemoglobin adducts in nonsmoking pregnant women and to compare adduct levels in those women with levels in smoking pregnant women.** ***Methods:*** **A questionnaire on smoking and exposure to environmental tobacco smoke was administered to 15 pregnant women who smoked cigarettes and 40 who did not smoke. Exposure was quantified for 1 week with a personal diary and by air sampling with a monitor worn by each woman. The monitor collected nicotine by passive diffusion to a filter treated with sodium bisulfate, and the deposit on the filter was analyzed by gas chromatography. Aliquots of maternal blood and cord blood collected during delivery were analyzed for 4-ABP–hemoglobin adducts by gas chromatography with negative ion chemical ionization mass spectrometry.** ***Results:*** **The mean adduct level in smokers (184 pg of 4-ABP per gram of hemoglobin) was substantially higher than that in nonsmokers (22 pg/g). This difference was statistically significant. Among nonsmokers, the levels of 4-ABP adducts increased significantly with increasing environmental tobacco smoke level ($P = .009$). Those in the lowest exposure category (<0.5 μg/m³ weekly average nicotine) had median 4-ABP–hemoglobin adduct levels of 15 pg of 4-ABP per gram of hemoglobin, while those in the highest exposure category (≥2.0 μg/m³) had median levels of 26 pg/g. Nonsmokers in this study had a median adduct level of 20 pg/g, and smokers had a median level of 143 pg/g.** ***Conclusions:*** **4-ABP–hemoglobin adduct levels in nonsmokers were 14% of the levels in smokers, which is consistent with findings of 20% in two other studies. Nonsmokers may receive a nontrivial dose of carcinogens from environmental tobacco smoke proportional to their exposure to environmental tobacco smoke.** ***Implication:*** **The relationship between environmental tobacco smoke exposure and 4-ABP–hemoglobin adduct levels supports epidemiologic evidence that environmental tobacco smoke is carcinogenic to passive smokers. [J Natl Cancer Inst 85:474–478, 1993]**

Environmental tobacco smoke contains more than 20 identified carcinogens to which nonsmokers may be exposed and is the only known environmental source of one of these carcinogens, 4-aminobiphenyl (4-ABP). 4-ABP was found to be a potent bladder carcinogen in workers in the dye industry, where its use has been discontinued for decades (*1*). The emissions of 4-ABP are more than 30 times greater in sidestream smoke than in mainstream smoke (*2*); therefore, passive smokers might receive substantial doses of this carcinogen. 4-ABP bonds covalently with hemoglobin, and this adduct might serve as a surrogate marker for a biologically effective dose (*3*).

There are several advantages to studying hemoglobin adducts of carcinogens as potential biologic markers: 1) Hemoglobin is abundant and easily obtained; 2) carcinogen–hemoglobin adducts can be measured in less than 10 mL of blood; 3) hemoglobin alterations are not subject to specific enzymatic repair mechanisms as are DNA modifications; and 4) hemoglobin acts as a cumulative dosimeter capable of indicating dose over an extended period of time. Unlike cotinine, which has a relatively short half-life of 1-2 days (*4*), hemoglobin adducts, once formed in adults, remain in circulation through the life of a red blood cell, about 120 days.

In a previous study, we (*5*) reported finding 4-ABP–hemoglobin adducts in the cord blood of babies born to both nonsmokers and smokers. This observation indicates that 4-ABP or its metabolite crosses the human placenta and binds to fetal hemoglobin. The present study examines the relationship between quantitative measures of exposure to environmental tobacco smoke and the levels of 4-ABP–hemoglobin adducts in the same nonsmoking pregnant women reported in the previous study (*5*) and compares these levels of 4-ABP–hemoglobin adducts to the adduct concentrations found among the same smoking pregnant women reported in our previous study (*5*).

Subjects and Methods

Exposure Assessment

We measured exposure to environmental tobacco smoke by using the following three tools

*See "Notes" section following "References."

that have been described in detail previously (6-8): 1) At enrollment, the subject was asked to complete a detailed questionnaire, administered by an investigator, on passive smoking exposure during an average week and was asked a subset of questions on two later occasions to evaluate how exposures had changed; 2) the subject was asked to complete a 7-day diary of environmental tobacco smoke exposure; and 3) during the same week that the subject was completing the diary, the subject was asked to wear a passive monitor to sample the air for environmental tobacco smoke.

Both the full questionnaire and the 7-day daily diary collected detailed information on environmental tobacco smoke exposure. This information included the location of the exposure (home, work, car, public place), the number of smokers present, the duration of the exposure, the distance of the subject from the smokers, and a subjective rating of the intensity of the environmental tobacco smoke. All women answered all questions. The questionnaires and the diaries were scored in the same manner as detailed previously (7); i.e., cumulative environmental tobacco smoke exposure = sum of all exposures (number of hours × number of smokers × proximity factor), where the proximity factor is 1 when smokers were more than 1.8 m (>6 feet) from the subject, 2 for smokers within 0.6-1.8 m (2-6 feet), and 3 when the smokers were within less than 0.6 m (<2 feet). Where there were too many smokers to estimate the number, e.g., in public places or at parties, an estimate of the intensity was substituted.

Air sampling of weekly exposure to environmental tobacco smoke was based on the collection of nicotine as a marker for environmental tobacco smoke (8-10). Each subject wore a lightweight (15 g) monitor that sampled nicotine by passive diffusion to a filter treated with sodium bisulfate. Subjects recorded in their diary the times they were not able to wear the monitor. The nicotine was desorbed from the filters, the pH was adjusted to above 9, and the nicotine was extracted into heptane and analyzed by gas chromatography as described previously (6).

The weekly average nicotine concentrations during the third trimester were used to categorize environmental tobacco smoke exposures in the analysis of data. This measure was thought to be less subjective than the diary and questionnaire. The cumulative exposures to environmental tobacco smoke calculated from the diary and the questionnaire (above), however, were each well correlated with the weekly average nicotine concentration (r = .87 and r = .86, respectively).

Although environmental tobacco smoke is the only identified source of 4-ABP in the nonoccupational environment, 4-nitrobiphenyl, which forms the same hemoglobin adduct and hence is an interferent, is produced by kerosene heaters and gas stoves. Therefore, the questionnaire included questions on the presence and use of gas stoves, kerosene heaters, wood-burning stoves, and fireplaces in the home.

Subject Enrollment

Women attending the prenatal clinic at the Medical Center of Central Massachusetts—Memorial (Worcester, Mass.) were recruited for this study during the second or third trimester of their pregnancies. The study was explained to them, and those who chose to participate signed consent forms approved by the University of Massachusetts Medical Center Human Subjects Committee. Altogether, 20 smoking women and 54 nonsmoking women were enrolled in the study. Smoking subjects were enrolled during the third trimester of pregnancy.

Initially, nonsmoking subjects were recruited in the second trimester and asked to wear the nicotine-sampling monitor and to complete the weekly diary twice (i.e., at enrollment and in the third trimester). Another questionnaire was administered during the third trimester to determine if changes in exposure had occurred. Some subjects, however, were less willing to participate the second time. For eight of those subjects who did participate both times, the environmental tobacco smoke exposures were found to have dropped in the third trimester as certain activities such as work were discontinued. Given the 120-day life span of adult red blood cells, exposures in the third trimester will have a greater impact on the levels of 4-ABP–hemoglobin adducts at the time of delivery. Therefore, 14 nonsmoking subjects recruited later in the study were asked to wear the nicotine-sampling monitor and to complete the diary only during the third trimester.

The results of the third trimester exposure were used to evaluate the relationship between environmental tobacco smoke exposure and 4-ABP–hemoglobin adducts at delivery. For those subjects who wore the monitor only during the second trimester, their responses to the questionnaire administered during the third trimester were used to evaluate whether a change in environmental tobacco smoke exposure had occurred; if a change had occurred, the concentration of nicotine to which a woman was exposed was assumed to have changed proportionately. For example, if work exposure accounted for 40% of the environmental tobacco smoke-exposure score in the second trimester (as determined from the questionnaire and diary) and if the questionnaire indicated that during the third trimester the subject discontinued work but the home and other exposures did not change, the third trimester exposure was estimated to be 60% of that measured in the second trimester. These adjustments were made without knowledge of the 4-ABP–hemoglobin adduct results and were applied to 12 subjects.

Collection of Blood

During delivery, 10-20 mL of maternal blood and 10-20 mL of cord blood were collected in heparin and then stored in a refrigerator. Within 48 hours of delivery, the blood was shipped on ice to the laboratory at the Massachusetts Institute of Technology. Blood samples were not collected for three (25%) of the 12 nonsmokers with the highest level of exposure to environmental tobacco smoke (≥2 μg/m³ nicotine) and for one (12.5%) of eight nonsmokers in the lowest exposure category (<0.5 μg/m³). Reasons identified for missed blood collections among smokers included a change of health-care provider (four), refusal (one), oversight on the part of hospital personnel and an unexplained change in chart retrieval mechanism on the labor and delivery unit (four), and overriding obstetrical emergencies (five).

4-ABP Adducts of Hemoglobin

The procedure used in this study was essentially the same as that reported previously (3,5). Briefly, the packed red blood cells from 10 mL of blood were washed with a saline solution and lysed with a combination of distilled water and toluene. The supernatant after centrifugation was dialyzed against distilled water and was used directly for analysis. Hemoglobin content was determined by Drabkin's assay (procedure No. 525; Sigma Chemical Co., St. Louis, Mo.), and the internal standard (a solution of hemoglobin previously adducted with *N*-hydroxy-4-aminobiphenyl-d_9 and containing 150 pg of hydrolyzable amine) was added. After 30 minutes at room temperature, sufficient 10 *M* NaOH was added to make the mixture 0.1 *M* NaOH. The amines were extracted with hexane after 1 hour and derivatized with pentafluoropropionic anhydride. The hexane solution was concentrated to 20 μL for analysis by capillary gas chromatography with negative ion chemical ionization mass spectrometry. Selected ion monitoring for the derivatives of the amines could detect less than 10 pg of 4-ABP adduct per 10 mL of blood. The assay has a precision of 4% when the deuterated adduct standard is used.

Results

Fifty-four pregnant nonsmokers and 20 pregnant smokers were enrolled in this study. An unknown interference prevented the analysis of the blood from one nonsmoker. Blood was collected and analyzed for hemoglobin adducts of 4-ABP for 40 of the nonsmokers and 15 of the smokers. The mean level of adducts among the 15 smokers was 184 pg of 4-ABP per gram of hemoglobin, while that for the 40 nonsmokers was 22 pg of 4-ABP per gram of hemoglobin (Table 1). Other studies have reported similar, though slightly lower, values for smokers, e.g., means of 154 (3) and 155 and 139 (11) pg of 4-ABP per gram of hemoglobin, and values slightly higher than those reported here for nonsmokers, e.g., 28 (3), 32 and 36 (11), and approximately 50 (12) pg of 4-ABP per gram of hemoglobin. In this study and in previous studies (3,11), there was no overlap between the values found for smokers and nonsmokers. The actual levels found in any

Table 1. 4-ABP–hemoglobin adduct levels in smokers and nonsmokers

	No.	Picograms of 4-ABP per gram of hemoglobin			
		Range	Median	Mean	SE
Nonsmokers	40	11-48	20	22	1.3
Smokers	15	50-419	143	184	28.1

population will depend on the smoking habits of smokers and the passive smoking exposures of nonsmokers, as well as on individual metabolic differences.

To examine whether there was any relationship between the environmental tobacco smoke exposures and the levels of 4-ABP–hemoglobin adducts in nonsmokers, we categorized average nicotine exposure in the third trimester into low, moderate, and high environmental tobacco smoke exposure groups and calculated the median and mean adduct levels in each category (Table 2). Four of the nonsmoking subjects for whom levels of 4-ABP–hemoglobin adducts were determined did not have measurements of personal nicotine exposure; therefore, they were omitted from this analysis. As environmental tobacco smoke exposure increased, so did both the median (from 15 to 17 to 26 pg of 4-ABP per gram of hemoglobin) and the mean (from 17.6 to 20.8 to 27.8 pg of 4-ABP per gram of hemoglobin) 4-ABP–hemoglobin adduct levels. The adduct levels were regressed against the exposure categories ($P = .009$). An analysis of variance also revealed that this relationship was statistically significant ($P = .027$). Post hoc analysis by the Tukey HSD test (*13*) indicated that, although the lowest two categories of environmental tobacco smoke exposure did not differ significantly from each other, the two highest categories differed ($P = .07$), and the highest and the lowest differed significantly ($P = .03$) from each other. The relationship between environmental tobacco smoke exposure and adduct levels was stronger in this study than in other studies (*11,12*), probably because we used more complete and objective measures of exposure.

Kerosene heaters and gas burners emit 4-nitrobiphenyl (*14*), which is a positive interference in the analysis of 4-ABP–hemoglobin adducts. We, therefore, re-examined the relationship between environmental tobacco smoke exposure and 4-ABP–hemoglobin adduct levels after we excluded subjects with kerosene heaters, gas stoves, wood-burning stoves, or fireplaces in their homes. Among the 40 nonsmoking subjects, none lived in homes that used kerosene heaters or wood-burning stoves or fireplaces; 14 subjects used gas stoves, 23 did not, and no information was available for the other three subjects. The relationship between environmental tobacco smoke exposure and 4-ABP–hemoglobin adduct levels remained after subjects with gas stoves were excluded (Table 2).

Blood was not collected from 13 nonsmoking subjects in this study. Of the five nonsmoking subjects for whom obstetrical emergencies prevented the collection of blood, one was in the lowest exposure quartile (concentration of nicotine, <0.5 $\mu g/m^3$) and three were in the highest exposure quartile (concentration of nicotine, ≥ 2.0 $\mu g/m^3$).

Discussion

Biologic indicators of exposure, such as urinary cotinine, have often been suggested as estimates of dose or, in the case of active and passive smoking, as estimates of the equivalent number of cigarettes smoked by a nonsmoker. However, one should be cautious in using these indicators, particularly in the case of complex mixtures such as environmental tobacco smoke. Nonsmokers in this study had 14% as much 4-ABP–hemoglobin adduct as smokers; two other studies (*3,11*) have reported 20%. However, the level of cotinine, a metabolite of nicotine, is generally two orders of magnitude higher in smokers than in nonsmokers (*15,16*).

That nonsmokers appear to have approximately 10%-20% the 4-ABP–hemoglobin adduct level as smokers may at first seem to be contradictory to the urinary cotinine ratios of about 1%, but in fact both results are quite consistent with our knowledge of the emissions of various contaminants in mainstream smoke and in sidestream smoke. Environmental tobacco smoke is a mixture of sidestream smoke (approximately 80%), emitted from the tip of the cigarette, and exhaled mainstream smoke. Although the same compounds are present in sidestream smoke (inhaled by the passive smoker) and in mainstream smoke (inhaled by the active smoker), their proportions are quite different (*17,18*). Approximately twice as much nicotine is emitted in sidestream smoke as in mainstream smoke, but about 31 times as much 4-ABP is emitted in sidestream smoke as in mainstream smoke (*2,17*). Thus, compared with mainstream smoke, sidestream smoke is 15

Table 2. Levels of 4-ABP–hemoglobin adducts in nonsmokers with varying exposures to environmental tobacco smoke

Weekly average nicotine concentration, $\mu g/m^3$	Picograms of 4-ABP per gram of hemoglobin			
	No.	Median	Mean	SE
	*All nonsmokers**			
<0.5	7	15	17.6	2.4
0.5-1.9	20	17	20.8	2.0
≥ 2.0	9	26	27.8	1.4
	Excluding nonsmokers with gas stoves			
<0.5	5	17	18.6	3.3
0.5-1.9	13	17	19.4	2.6
≥ 2.0	6	27	28.2	1.6

*Four of the nonsmoking subjects for whom levels of 4-ABP–hemoglobin adducts were determined did not have measurements of personal nicotine exposure; therefore, they are omitted from this analysis.

times more enriched in 4-ABP than in nicotine, and the ratio of biomarkers in those nonsmokers exposed to environmental tobacco smoke compared with smokers is 15 times greater for the biomarker 4-ABP–hemoglobin adducts than for the biomarker cotinine, a metabolite of nicotine. Therefore, the "cigarette equivalent" dose (i.e., the number of cigarettes one would have to smoke to receive the same amount of the chemical) of those nonsmokers exposed to environmental tobacco smoke varies with the compound. As a result, a passive smoker may receive 1% as much nicotine as an active smoker, but 15% as much 4-ABP. The actual percentages would, of course, vary with each individual's actual exposure to environmental tobacco smoke.

One should also exercise caution in extrapolating the findings from this study to other settings of environmental tobacco smoke exposure, such as restaurants, bars, offices, or homes, because of the importance of the time during which exposures were averaged. In studies of particular environments of interest, measurements are made during the time an individual spends in the environment. To conduct a study comparable to this study, one would have to measure *all* environments in which an individual spent time, including the bedroom during sleep, and calculate a time-weighted average for the entire week. In this study, subjects wore the nicotine-sampling monitor while they were awake and kept it in the bedroom (e.g., placed it on a bureau or nightstand) while they slept; this routine was carried out for a full week. The exposures of the nonsmokers reported here are the weekly average nicotine exposures (i.e., averaged through seven 24-hour days); therefore, the exposures, when they occurred, were much higher than the weekly average values. For example, a person whose weekly average nicotine concentration was 0.5 $\mu g/m^3$, but who was exposed only 2 hours a day, would experience an average exposure of 6 $\mu g/m^3$ during those episodes and zero exposure during the other 22 hours of each day. Conversely, a person exposed to environmental tobacco smoke only at work, for 40 hours per week, at 10 $\mu g/m^3$, would have a weekly time-weighted average exposure of 2.4 $\mu g/m^3$.

A statistically significant relationship ($P = .009$) was found between the weekly average exposure to environmental tobacco smoke during the third trimester of pregnancy and the levels of 4-ABP–hemoglobin adducts found at the time of delivery. This relationship remained after subjects with possible exposure to gas stoves, a possible interference, were removed from the dataset. This result indicates that exposure to environmental tobacco smoke is related to levels of a known human carcinogen in nonsmokers. The increase is not dramatic, and the public health significance is unclear. The following facts, however, indicate that nonsmokers may receive a nontrivial dose of carcinogens from environmental tobacco smoke: 1) The carcinogen 4-ABP was detected in passive smokers at 14% the level found in smokers; 2) this ratio is consistent with the emissions of mainstream smoke and of sidestream smoke; and 3) greater amounts of other carcinogens, e.g., nitrosamines, are also emitted in sidestream smoke than in mainstream smoke. Although bladder cancer among passive smokers has not been studied, the incidence of lung cancer has been reported to be elevated among nonsmokers married to smokers (*17*). The relationship between environmental tobacco smoke exposure and 4-ABP–hemoglobin adduct levels contributes to the plausibility of epidemiologic evidence that environmental tobacco smoke is carcinogenic to passive smokers.

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Author affiliations: S. K. Hammond, J. Coghlin, P. H. Gann, M. Paul, Department of Family and Community Medicine, University of Massachusetts Medical Center, Worcester, Mass. K. Taghizadeh, P. L. Skipper, S. R. Tannenbaum, Department of Chemistry and Division of Toxicology, Massachusetts Institute of Technology, Cambridge, Mass.

Correspondence to: S. Katharine Hammond, Ph.D., Department of Family and Community Medicine, University of Massachusetts Medical Center, 55 Lake Ave. N., Worcester, MA 01655.

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Restoration of Taxol Sensitivity of Multidrug-Resistant Cells by the Cyclosporine SDZ PSC 833 and the Cyclopeptolide SDZ 280-446

*Bénédicte Jachez, Rene Nordmann, Francis Loor**

Background: **Taxol, a promising agent for the treatment of cancer, has entered phase II clinical trials. Nevertheless, it belongs to the class of compounds that show impaired retention in multidrug-resistant cells expressing P-glycoprotein (Pgp), a drug efflux pump. Chemosensitizers like verapamil modulate multidrug resistance by interfering with the efflux action of Pgp and thus can decrease drug resistance or can restore drug sensitivity by restoring normal drug accumulation and distribution within the multidrug-resistant tumor cell. The two strongest, nearly equipotent chemosensitizers identified to date are the cyclosporine derivative SDZ PSC 833 and the semisynthetic cyclopeptolide SDZ 280-446.** ***Purpose:*** **This study was designed to investigate the capacities of verapamil, SDZ PSC 833, and SDZ 280-446 to decrease resistance of two multidrug-resistant cell lines to taxol.** ***Methods:*** **We studied in vitro the growth of two multidrug-resistant tumor cell lines displaying high resistance to taxol: multidrug-resistant Chinese hamster ovary cells and murine monocytic leukemia P388 cells. We determined the taxol concentration that produced 50% inhibition of cell growth (IC_{50}) in the two multidrug-resistant cell lines and in the parent cell lines, in the presence of a range of chemosensitizer concentrations (0-30 μM). IC_{50} values were determined in the presence and in the absence of verapamil, SDZ PSC 833, or SDZ 280-446.** ***Results:*** **At nontoxic concentrations (0.3-1 μM), SDZ PSC 833 and SDZ 280-446 produced an almost complete reversal of the high taxol resistance of the multidrug-resistant tumor cells, whereas only partial restoration of sensitivity to taxol was achieved with verapamil.** ***Conclusion:*** **SDZ PSC 833 and SDZ 280-446 can restore the normal taxol sensitivity of highly resistant multidrug-resistant tumor cells.** ***Implications:*** **The combination of taxol with SDZ PSC 833 or SDZ 280-446 may be recommended for treatment of multidrug-resistant cancers. [J Natl Cancer Inst 85:478–483, 1993]**

Taxol (NSC-125973) is a new anticancer agent (*1,2*) presently undergoing phase I and II clinical trials (*3,4*). This drug is isolated from trees of the genus *Taxus*, and its ability to arrest cell proliferation in the G_2 and M phases of the cell cycle is well documented (*5,6*). This capacity correlates with its unusual properties of interaction with tubulin in vitro (hyperstabilization of microtubules) (*1*).

Unfortunately, a major mechanism of cellular resistance to taxol is the increased drug efflux out of cells that display the "classic" multidrug-resistant phenotype. An increased capacity to pump out a variety of drugs with unrelated structures and intracellular targets (e.g., vinca alkaloids, doxorubicin, etoposide, dactinomycin, and colchicine) correlates with increased levels of mdr gene-encoded membrane glycoproteins (P-glycoprotein) (*7,8*).

A variety of agents can decrease in vitro the drug resistance of multidrug-resistant tumor cells and sometimes completely restore their sensitivity to chemotherapeutic agents. Such chemosensitizers belong to various structural classes. By interfering with the efflux function of P-glycoprotein (Pgp), they can decrease drug resistance or can restore drug sensitivity by restoring the normal drug accumulation and distribution within the multidrug-resistant tumor cell (*7,8*). Among these chemosensitizers, the calcium channel blocker verapamil (*7*) has been the most commonly studied. Nevertheless, very potent chemosensitizers have been most commonly identified among cyclosporines (*9,10*) and cyclopeptolides (*11*) derived from a Fungi imperfecti (*Septoria* sp.). Extensive studies of chemically modified derivatives of cyclosporines and cyclopeptolides led to the selection of the two strongest, nearly equipotent chemosensitizers identified to date: the cyclosporine derivative SDZ PSC 833 and the semisynthetic cyclopeptolide SDZ 280-446 (*11,12*).

How taxol is processed by Pgp is unknown, and whether SDZ PSC 833 and SDZ 280-446 could restore a normal sensitivity to taxol was not predictable. The present study was, thus, aimed at (*a*) measuring the level of taxol resistance displayed by two multidrug-resistant tumor cell lines extensively studied in our laboratory and (*b*) testing whether taxol resistance could be modulated by verapamil, SDZ PSC 833, and SDZ 280-446.

Materials and Methods

Cell Lines

Parental and multidrug-resistant Chinese hamster ovary (CHO) cells (*13*) were obtained from Dr. V. Ling (Ontario Cancer Institute, Toronto, Canada). Parental and multidrug-resistant murine monocytic leukemia P388 cells (*14*) were obtained from Dr. M. Grandi (Farmitalia Carlo Erba Research Center, Milan, Italy). The multidrug-resistant P388 cell line has been selected by growth in the presence of doxorubicin for the multidrug-resistant phenotype (*14*). The multidrug-resistant CHO cell line has been selected by growth in the presence of colchicine for the multidrug-resistant phenotype (*13*). Detailed information on the properties of these cell lines (i.e., sensitivity to various drugs and effects of various chemosensitizers) has been published by our laboratory (*9-12*).

*See "Notes" section following "References."